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EPIDERMAL STRUCTURAL PROTEINS

III. ISOLATION AND PURIFICATION OF HISTIDINE-RICH PROTEIN OF THE NEWBORN RAT*

TADASHI TEZUKA, M.D., PH.D., AND IRWIN M. FREEDBERG, M.D.†

ABSTRACT

Granular cells from newborn rat epidermis have been extracted with deoxycholate and the extract chromatographed sequentially on Sephadex G-200 and G-75. A protein fraction has been obtained which is electrophoretically homogeneous. It has an amino acid composition which is high in histidine, glutamic acid, and glycine and low in leucine. The protein contains newly-incorporated radioactively labeled amino acids and is antigenic in rabbits. The material corresponds to the histidine-rich protein isolated with different techniques by other workers.

Autoradiographic studies have indicated that ^{3}H-labeled histidine and serine are preferentially incorporated into the granular cell layer in newborn rat epidermis [1–3]. In electron microscopical investigations, two-thirds of the ^{3}H-histidine grains were located on keratohyaline granules 6 hr following injection, although only one-third of the grains were similarly localized at 1 hr [4]. Fukuyama and Epstein [5] extracted glutaraldehyde-fixed epidermis with 0.1 N NaOH and found a 12:1 ratio of ^{3}H-histidine to ^{3}H-leucine in the solubilized extract. Since the alkaline extract removes material which was in the keratohyaline granules, these data indicate that components with a high histidine:leucine ratio are found in the granules.

Hoober and Bernstein [6] and Bernstein et al [7] isolated a histidine-rich protein from newborn rat epidermis and found that it was extractable with 0.11 N perchloric acid (PCA) from material which had been previously solubilized with 8 M urea. The PCA-soluble material had 7 histidine residues per 100 residues, was retarded on Sephadex G-100 column chromatography, and its molecular weight was estimated to be approximately 30,000. In an in vitro system Sugawara and Bernstein [8] found that ^{14}C-histidine was incorporated rapidly into a 0.1 N PCA soluble, pH 4.5 insoluble, protein and they proposed that synthesis of this protein involved at least two steps.

Ugel [9] isolated keratohyaline granules from cow-hoof epidermis and found a fraction of molecular weight 20,500 which contained 7 histidine residues per 100 residues. Matoltsy and Matoltsy [10], in contrast, extracted keratohyaline granules from newborn rat epidermis and found only a 3% histidine content. In previous studies reported from our laboratories [11], we used deoxycholate to extract material of keratohyaline origin from the granular layer of newborn rat epidermis. When ^{14}C-leucine and ^{3}H-histidine were injected into the rats, both amino acids could be found in proteins derived from the keratohyaline granules. Amino acid analysis of the material from the granules revealed that 3% of the amino acid residues were histidine [11].

The purpose of this report is to describe the isolation of another purified fraction from this same deoxycholate extract which has an amino acid composition similar to that of the material isolated by Bernstein and his co-workers.

EXPERIMENTAL PROCEDURES

Materials. Newborn rats (Sprague–Dawley strain; 3–4 days old) were used. Radioisotopes were purchased from New England Nuclear Corporation, Boston, Mass. (uniformly labeled L-^{14}C-leucine, 240 mCi/mmole; L-^{3}H-histidine, 6.24 mCi/mmole); sodium tetraphenylboron from Sigma Chemical Company, St. Louis, Mo.; sodium deoxycholate from Difco Laboratories, Detroit, Mich.; Sephadex G-200 and G-75 from Pharmacia Fine Chemicals, Inc., Fair Lawn, N.J. All other chemicals were of reagent or analytic grade obtained from various commercial sources.

Preparation and extraction of tissue. Epidermis was separated from dermis after a 30-min incubation in buffer containing 0.03 mM sodium tetraphenyboron (10 mM Tris-HCl, pH 8.8, 1 mM magnesium acetate, 10 mM NaCl, 5 mM 2-mercaptoethanol, 250 mM sucrose). Details have been described previously [11]. Epidermal sheets consisting of granular and horny cell layers were obtained by further incubation in tetraphenylboron for appropriate periods of time. Buffer changes were made every 24 hr and each extract was analyzed by light microscopy. The epidermal sheets were collected and manually minced. The tissue was subsequently homogenized (Kontes glass homogenizer) in the presence of 50 mM Tris-HCl (pH 8.8) containing 10 mM 2-mercaptoethanol and 3 mM sodium deoxycholate and stirred for 48 hr in the same solution.

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* From the Department of Dermatology, School of Medicine, Tokyo Medical and Dental University (Professor Dr. Saburo Kagawa), Tokyo, Japan, and † the Department of Dermatology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts. (Reprint requests to Dr. I. M. Freedberg, Beth Israel Hospital, 330 Brookline Avenue, Boston, Mass. 02215.)

The homogenate was centrifuged at 15,000 × *g* for 10 min (Tominaga refrigerated automatic centrifuge), and the resulting pellet was rehomogenized and stirred for an additional 48 hr. This procedure was repeated three times. The 15,000 × *g* supernatant fractions obtained at each 48-hr period were recentrifuged at 270,000 × *g* for 1 hr (Beckman-Spinco Model L-2-65). The lipid layer was discarded and the high-speed supernatant fraction was removed, dialyzed against 10 mM Tris–HCl (pH 8.8) for 24 hr, concentrated by lyophilization, and stored at −80°C.

Gel filtration studies. The deoxycholate-soluble material was dissolved in 50 mM Tris–HCl (pH 8.8), made 2% with respect to deoxycholate, and was subjected to gel filtration on a column (2.5 x 34 cm) of Sephadex G-200. The elution profile was monitered at 280 nm during elution with 50 mM Tris–HCl (pH 8.8) containing 10 mM 2-mercaptoethanol. Of the several peaks described below, the shoulder region, which eluted following the plateau region, was collected, dialyzed, and lyophilized. This fraction was further chromatographed on a Sephadex G-75 column (2.5 x 100 cm) using the same eluant. Protein content was determined by the technique of Lowry et al [12].

Gel electrophoresis. Various fractions were subjected to disc gel electrophoresis using 10% acrylamide in the presence of sodium dodecylsulfate (SDS) according to the technique of Weber and Osborn [13].

Isotopic incorporation studies. Prior to experiments using radioactively labeled proteins, newborn rats were injected intracutaneously or intraperitoneally with 1 μCi of ^{3}H-histidine and 1 μCi of ^{14}C-leucine. These rats were killed at various times following injection (3–24 hr). The tissues were fractionated in a manner identical to that described above. Aliquots of the gel filtration eluates were made 10% with respect to trichloroacetic acid, and the proteins and nucleic acids were allowed to precipitate overnight at 4°C. The precipitates were suspended in 5% trichloroacetic acid and washed 4 times in the same solution, collected on 0.45-μ Millipore filters, and dried. Radioactivity was determined by scintillation counting in a Packard Tricarb Spectrometer. All data were corrected for background and quenching.

Immunologic procedures. The lyophilized, second peak from the Sephadex G-75 column was dissolved in 50 mM Tris–HCl (pH 8.8), emulsified with an equal amount of Freund's complete adjuvant, and injected into foot pads of rabbits (1 mg of protein in a 2.0-ml emulsion was injected into each animal). Sera were assayed for precipitating antibody content using the Ouchterlony double-diffusion technique [14]. One percent agar in 50 mM Tris–HCl buffer (pH 7.3) was used. Immunoelectrophoresis [15] was carried out in a barbituric acid–sodium barbiturate mixture at 0.1 ionic strength and pH 8.6. The support, 2 ml of 2% agar at pH 8.6, was deposited with a pipette on a thoroughly cleaned and dried microscope slide. Electrophoresis was performed for 60 min at 40 volts. The antigen–antibody reactions were developed in a humid chamber at room temperature overnight.

Amino acids analysis. Amino acid analyses were obtained following hydrolysis at 100°C in vacuo for 18 hr in the presence of 6.0 M redistilled hydrochloric acid. Analyses were performed on a Beckman automatic amino acid analyzer equipped with an automatic integrator.

RESULTS

Isolation of Histidine-rich Fraction

The isolation of epidermal sheets consisting of only granular and horny cell layers was achieved by incubation in Tris buffer containing tetraphenylboron [11]. From this pretreated epidermis, following homogenization and incubation in deoxycholate, subcellular organelles, including keratohyaline granules, ribosomes, and the interfilamentous matrix can be extracted. The extraction process has been documented by electron microscopic techniques in a previous paper [11]. The deoxycholate-soluble fraction was subjected to further centrifugation in order to free the solubilized keratohyaline and its precursors from any ribosomes, vesicles, and mitochondria.

The 270,000 × *g* supernatant fraction was subjected to column chromatography on Sephadex G-200 (Fig. 1). Deoxycholate-soluble material was eluted at the void volume followed by a plateau region, an ascending shoulder, and a final nonprotein peak which contained material with 280 absorbancy.

The proteins in the initial peak, plateau region, and the shoulder area of the final peak were quantitated by the procedure of Lowry (Table I).

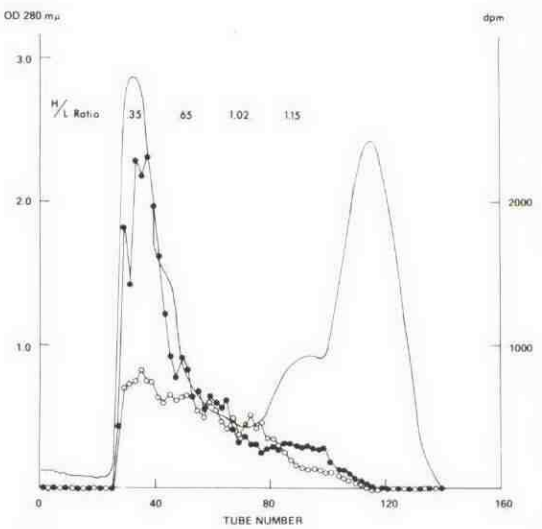

FIG. 1: Chromatography of deoxycholate-soluble supernatant fraction on Sephadex G-200. The data are an average of 4 experiments. —— = optical density at 280 nm; ●——● = ^{3}H-histidine incorporation; O——O = ^{14}C-leucine incorporation; H/L ratio = ratio of incorporated histidine/leucine in pooled chromatographic tubes 27–45, 46–60, 61–80, and 81–90. (Figure modified from [11].)

TABLE I

Protein content of fractions from Sephadex G-200 column

Fraction	A		B		C	
Chromatographic Tubes	27–50		51–80		81–90	
	mg	%	mg	%	mg	%
Exp. I	42	23.1	64	35.2	76	41.8
Exp. II	60	27.4	78.4	35.8	80.8	36.9
Exp. III	38	35.5	31	29.0	38	35.5

In these separate experiments, around one-fifth of the total protein (18–27%) was eluted in the shoulder area (*C*). Samples from this area of the chromatogram were pooled, dialyzed, lyophilized, and rechromatographed on Sephadex G-75 with the elution pattern presented in Figure 2. The initial peak at the void volume was followed by a second peak and a final, predominantly nonprotein peak. When 75 mg of the material from the initial shoulder were placed on the Sephadex G-75 column, the protein contents of the three resulting peaks were 28.8, 46.4, and 5.4 mg, respectively. Essentially 100% recovery of protein was achieved.

Incorporation Studies

In our previous studies [11], 1 μCi of ^{3}H-histidine and 1 μCi of ^{14}C-leucine were injected intracutaneously into rats, the animals were killed at 16 hr following injection and the epidermis was fractionated as described above. Approximately 50% of the incorporated histidine and 30% of the incorporated leucine were found in the deoxycholate-soluble, 270,000 $\times$ *g* supernatant fraction of the epidermal homogenate. The distribution of incorporated amino acids in the 270,000 $\times$ *g* supernatant fraction was further analyzed by molecular sieve chromatography on Sephadex G-200. The highest total histidine incorporation was found in the initial peak, but the relative incorporation of histidine compared to leucine was greatest at the beginning of the shoulder area, between chromatographic tubes 80 and 90 (Fig. 1).

In order to gain information concerning the rate at which the material in this portion of the chromatogram was synthesized in vivo, 1 μCi of ^{3}H-histidine and 1 μCi of ^{14}C-leucine were injected intraperitoneally into 30 rats. Five rats were killed at various periods of time from 3 to 24 hr and the deoxycholate-soluble material was extracted as described above. The 270,000 $\times$ *g* supernatant fractions were subjected to column chromatography on Sephadex G-200 (1.0 x 50 cm column) and the proteins precipitable in 10% trichloroacetic acid from the initial peak and the shoulder area were analyzed. The specific activity of ^{3}H-histidine in the proteins of the shoulder area reached a maximum 15 hr following injection and the specific histidine radioactivity remained 2 times higher

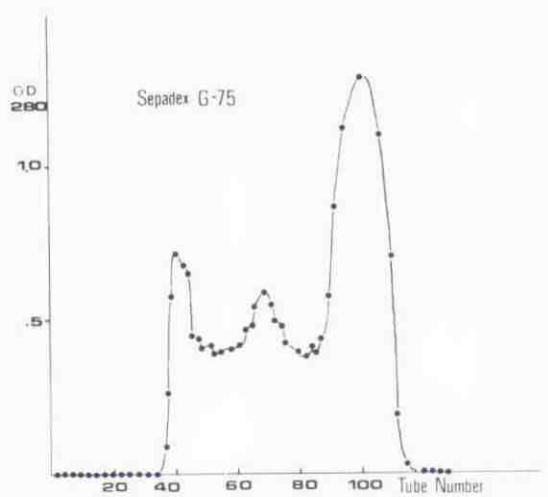

FIG. 2: Molecular sieve chromatography (Sephadex G-75) of the shoulder area from the Sephadex G-200 column. Three-milliliter fractions were collected. ●——● = optical density at 280 nm.

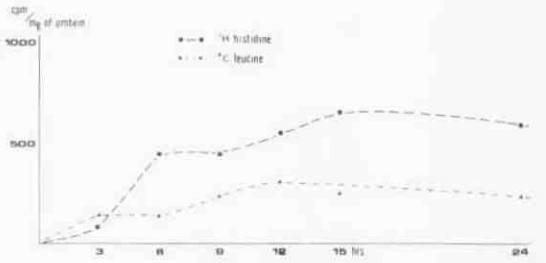

FIG. 3: Incorporation of ^{3}H-histidine and ^{14}C-leucine into proteins of pooled tubes 81–90 from the Sephadex G-200 column. ●——● = ^{3}H-histidine incorporation; ▲——▲ = ^{14}C-leucine incorporation.

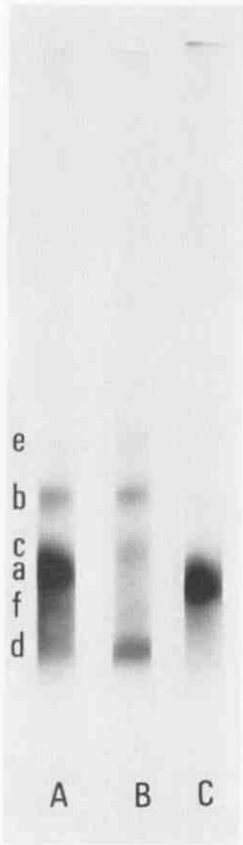

FIG. 4: Acrylamide gel electrophoretic patterns of the deoxycholate-soluble material. Origin is at the top in each case. Gels *A*, *B*, and *C* were run in the presence of sodium dodecylsulfate; 47 μg of protein of the shoulder region from the Sephadex G-200 column (*A*), 48 μg of the initial peak (*B*), and 23 μg of the second peak from the Sephadex G-75 column were applied to the gels. Identified bands are noted by letters on the left.

than that of ^{14}C-leucine throughout the experimental period from 6 hr (Fig. 3).

Electrophoretic Studies (Fig. 4)

Acrylamide gel electrophoresis in the presence of sodium dodecylsulfate of the shoulder region from the Sephadex G-200 chromatogram revealed 1 major, 3 minor, and 2 very faint protein bands (*A*). When the material from the shoulder region was rechromatographed on Sephadex G-75, the same major band was found in electrophoretograms of the second peak (*C*) but not in those of the first peak (*B*). The major protein band could thus be isolated from the several other components in the starting material. The fact that faint bands could still be observed in gel *C* indicates that total purification was not accomplished.

Amino Acid Analyses

In Table II are displayed the results of amino acid analyses performed on aliquots from the initial and second peaks of the Sephadex G-75 chromatogram. The results of Hoober and Bernstein concerning histidine-rich protein are tabulated for comparison. The most common amino acid residues in the proteins of the material we have isolated are glutamic acid, glycine, and serine. Arginine and histidine contents are also relatively high in both protein-containing peaks from the G-75 column. In contrast, the leucine content is low.

Immunologic Studies

In order to determine the relationship between the proteins eluted from the Sephadex G-75 column and other fractions isolated from keratohyaline granules with deoxycholate, immunologic studies were carried out. Figure 5*A* indicates that the Sephadex G-75 second peak was antigenic in rabbits, eliciting a precipitating antibody which formed a single, sharp line of precipitation in agar when run against the immunizing antigen. Immunologic identity exists between the material of this second peak and the Sephadex G-200 shoulder fraction. No precipitin band was formed when the initial peak from Sephadex G-200 column was similarly analyzed.

Immunoelectrophoretic analysis (Fig. 5*B*) indicates that the major protein band of the second peak from the Sephadex G-75 column is, in fact, the antigenic substance. Analysis following SDS electrophoresis (Fig. 5*C*) has not indicated any minor contaminants which react with this antibody.

TABLE II

Amino acid analysis (residues/100 residues)

	I	II	*
Lys	6.4	6.6	1.0
His	7.2	10.2	6.9
Arg	9.5	9.0	9.0
Asp	7.1	7.3	5.6
Thr	5.5	5.9	6.8
Ser	11.6	10.6	11.5
Glu	10.6	13.2	14.1
Pro	5.2	5.2	—
Gly	11.8	11.5	15.3
Ala	8.4	6.6	11.8
Val	2.6	2.8	—
Met	1.1	1.2	—
Ile	2.8	2.4	—
Leu	3.7	3.0	—
Tyr	4.3	3.0	~3.0
Phe	2.2	1.6	—
Cys	—	—	—

I: the initial peak from the Sephadex G-75 column. II: the second peak from the Sephadex G-75 column. *: "histidine-rich protein" of Hoober and Bernstein.

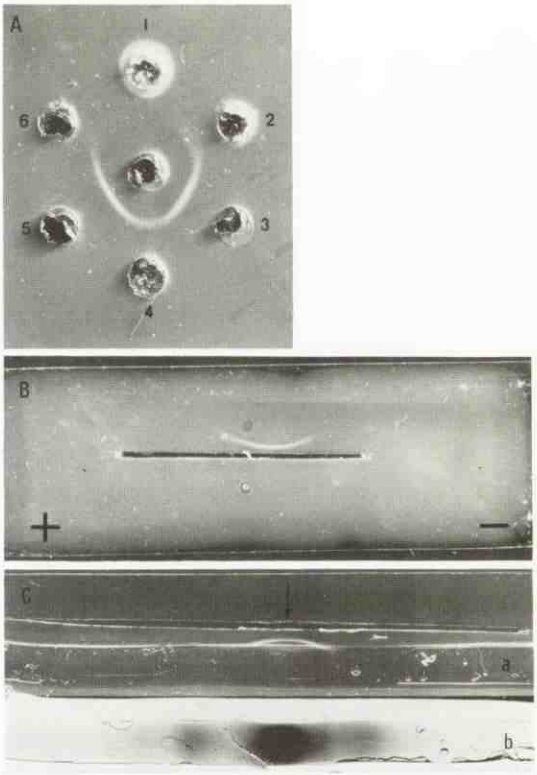

FIG. 5: *A*: Analysis by double diffusion in agar of antibody raised to the second peak from the Sephadex G-75 column. Center well: antibody; peripheral wells: 1–3 are aliquots of initial peak, plateau region, and shoulder region, respectively, of the G-200 column, and 4–6 contain aliquots of initial, second, and third peaks, respectively, of the G-75 column. *B*: Immunoelectrophoresis of second peak from G-75 column in upper well; normal rat serum in lower well. Antibody as above. *C*: Analysis of material from shoulder region of G-200 column by disc gel electrophoresis in sodium dodecylsulfate followed by immunoelectrophoresis. The precipitin reaction is seen in *A* and the stained protein band in *B*, the location of each marked by the arrow.

DISCUSSION

Previous studies from our laboratories have indicated that keratohyaline granules can be solubilized

with sodium deoxycholate [11]. Chromatography of the deoxycholate extract revealed a major protein band which could be used to raise antibodies in rabbits. The antibodies reacted, when studied by immunofluorescence, with in situ keratohyaline granules, and the protein used as antigen did not resemble in amino acid composition the protein which has come to be known as HRP or "histidine-rich protein."

HRP was initially isolated by Hoober and Berstein [6] using urea solubilization and subsequent perchloric acid extraction. Recent studies by Sibrack, Gray, and Bernstein [16] have indicated quite clearly that HRP is a component of keratohyaline granular material isolated from newborn rat skin following phosphate extraction.

The data in the present paper indicate that it is possible to isolate from deoxycholate extracts of keratohyaline granule origin a protein fraction which is high in histidine and arginine content and relatively low in leucine—characteristics similar to those of HRP. The fraction which was initially identified as a shoulder region in eluates from Sephadex G-200 columns, can be further purified by chromatography on Sephadex G-75. Electrophoretograms of material from the G-75 columns indicate that a major protein has been isolated which differs in amino acid composition, migration, and immunochemical reactivity from the fractions we have previously isolated from the keratohyaline granules.

The proteins discussed in the paper form a portion of the nascent protein pool of the epidermal cell of newborn rats as indicated by the fact that isotopically labeled amino acids become incorporated into the proteins rapidly. Our data, however, do not prove the relationship between these proteins and those isolated by other workers. They also do not indicate the true relationship, either structural or functional, of the several fractions which have been isolated from keratohyaline granules. These latter questions will be completely clarified only when the granules can be isolated in their intact form and analyzed in the several extraction conditions used by laboratories. It is in this direction that our current endeavors are proceeding.

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